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Identifying protein interactors for the cytoplasmic domain of *C. elegans* L1 cell adhesion molecule via yeast-two-hybrid screen

Overview

The purpose of this project was a discovery-based series of experiments designed to identify novel interactors with the cytoplasmic tail of the SAX-7 protein in *C.elegans*. SAX-7 is a homologue of the mammalian L1 cell adhesion molecule (L1CAM), a plasma membrane protein that regulates neurite outgrowth and adhesion (1). The significance of identifying L1CAM interactors has high-impact translational relevance to medicine, as faulty L1CAMs in humans can result in serious neurological CRASH conditions, well characterized symptoms constituting **C**orpus callosum hypoplasia, mental **R**etardation, **A**dducted thumbs, **S**pastic paraplegia, and **H**ydrocephalus (Chen, 2010). SAX-7 expression in *C. elegans* is expressed in neural and non-neuronal tissue, which would suggest this protein serves a different function than it does in humans. Nonetheless, L1CAM and SAX-7 share sequence homology in their cytosolic binding sites: FERM, PDZ, and ankyrin binding sites (Zhou, 2010). The protein interactors with SAX-7 are largely unknown, making it difficult to address the mechanisms by which SAX-7 and L1CAM are similar or dissimilar in stabilization at the plasma membrane, regulation, and physiological consequence.

Here we report the construction of a *de novo* PCR amplified *C. elegans* cDNA library, transformation into a yeast Y187 prey expression system, and mating screen performed with the SAX-7 protein expressed in complementary AH109 bait expression yeast. More than 4,000 individual colonies containing potential interactors were identified. The primary benefits of utilizing this mating protocol (compared to typical co-transformation assays) are that yeast mating facilitates a large scale interaction screen, and diploid cells have a reduced sensitivity to transcription activation compared to haploid cells resulting in reduced background (Koolonin, 2000). This report will detail methods that may be used to generate an equivalent *C.elegans* library and screens in order to validate the competency of the library, allowing for an unbiased screening of SAX-7 or other cytosolic proteins.

Methods and Results

Total RNA purification and Poly(a) RNA enrichment:

Wild type N2 *C.elegans* worms were plated on NGM (nematode growth medium) plates (8-12 worms per plate) covered in a thin layer of 2% agarose and seeded with OP50 *E.coli*. These worms were allowed to populate 8 plates for 5 days at 20 °C. Total RNA was extracted using Trizol (Life Technologies). Redacted protocol: worms were washed with 15 mL M9 solution and pelleted. 500 μL Trizol reagent and 100 μL sterile glass beads (1mm) were vortex with worms to break the waxy cuticle. Three rounds of freeze-thawing were performed using liquid nitrogen and a 37°C water bath; chloroform, acid phenol, chloroform again, followed by 1 μL of glycoblue and 700 μL of isopropanol. A wash with 70% ethanol was performed followed by resuspension of total RNA in 50 μL RNase free water. Full methods available from Cold Spring Harbor. Total RNA concentration was measured via nanodrop spectrophotometer (260/280 wavelength) and samples were tested for RNase degradation by running samples on a gel in order to inspect ribosomal RNA.

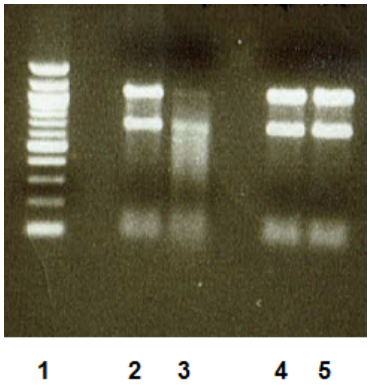

Figure 1: Testing total RNA for digestion by RNase.

Lane 1: 10 µL of 100bp ladder. Lane 2-5 are RNA samples at the following concentration: 2: 2836 ng/µL, 3:7296 ng/µL, 4: 7847 ng/µL, 5. 9014 ng/µL. Each sample contained 0.2 µL RNA, 4.8 DEPC water, and 5 µL loading buffer, loaded onto a 1% agarose 1xTAE/EtBr gel and imaged under UV light. Lane 3 demonstrates extensive digestion of ribosomal RNA bands. Sample 5, at the highest concentration, was selected for poly(A) enrichment.

As subsequent steps involved use of random hexamer primers for generating cDNA, it was required that we enriched the total RNA sample specifically for mRNA. We utilized the NucleoTrap mRNA Purification of poly(A) kit (Macherey-Nagel). However, due to low yields (<30 ng/µL), we modified protocols to include all optional washes as well as an additional RM3 wash. Additional washes assisted in removing mRNA binding proteins, allowing poly(A) RNA to be purified at a final yield of 91.6 ng/µL.

RT-PCR using SMART system and PCR amplification:

We generated reverse transcribed cDNA from poly(A) enriched mRNA as per the Make Your Own "Mate and Plate™" Library User Manual (Clontech). As we wanted to select for any potential protein interacting domain, and not just domains from the C-terminal end of proteins, random hexamer primers were used to generate the cDNA library rather than poly(A) primers. We modified protocols to use 3µL of poly(A) enriched mRNA, for a total of 274.8 ng being used to generate 10 µL of unamplified cDNA. Following construction of cDNA, we performed long distance PCR amplification as per instructions. We optimized PCR protocols, using 22 cycles, 24 cyles, and 26 cycles. As demonstrated in Figure 2, 24 cycles resulted in the optimum yield of cDNA extending past 4000kb. Selection of longer cDNA reduces excessive amplification of short sequences, which would skew library away from being an accurate representation of cellular mRNA transcripts.

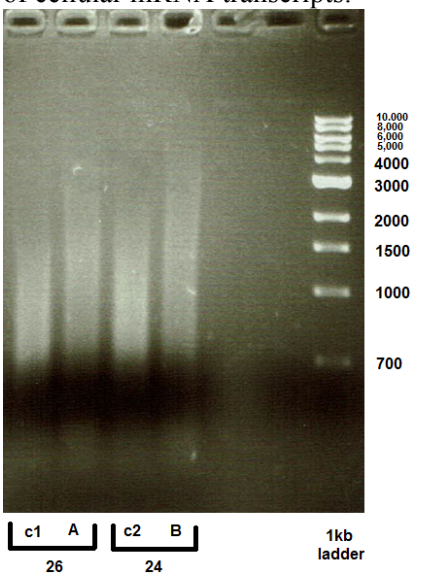

Figure 2: Results from LD-PCR amplification of generated cDNA.

7µL aliquots per well run alongside 0.25 µg 1 kb ladder on a 1.2% agarose/EtBr gel. The c1 and c2 lanes are mouse liver control, used to verify successful functioning of reverse transcription and PCR protocol. As shown, sample A was performed for 26 PCR cycles, resulting in fragments of 3kb in length. Sample B was run for 24 PCR cycles, resulting in fragments of 4 kb in length.

Resultant products from the PCR cycle had a DNA concentration *circa* 450 ng/µL, with 7 µL of 100 µL cDNA being used on a gel to test range of library. Thus, we had 93 µL for a total of 41 µg of DNA per PCR reaction available. In order to select for longer PCR fragments, we ran samples A and B (93 µL each) through a CHROMA SPIN™ +TE-400 column used to select for DNA molecules longer than

200bp (Clontech). Unfortunately, our total yield was only 1 μg cDNA coming off the CHROMA SPIN matrix, which is half the required minimum to proceed to transformation procedures (2-5 μg DNA). In order to come up with the required 2μg cDNA, we generated new cDNA by RT-PCR set for 24 cycles, resulting in 530 ng/μL cDNA extending to 3kb in length. Since we had used all 10 μL of our best sample of unamplified cDNA, we further extended our 22 cycle product from previous optimization protocol for 2 more cycles (resultant yield was sub-par, of only 2kb). Both the new 24 cycle generated and the 22+2 cycle products were run through CHROMA SPIN for a resultant yield of 1 μg cDNA. This sample was combined with the previously generated 1 μg cDNA for the requisite 2 μg needed.

Transformation optimization:

As we only had 2 μg of library available, extensive optimization of a small-scale transformation protocol was performed in order to ensure library scale transformation success. We transformed pGADT7-T prey plasmid, which expresses enzymes for the production of leucine as well as viral large T antigen fused to the 3' end of the Gal4 AD (activation domain), into Y187 yeast strain which specifically does not express leucine (nor other reporter markers) and plated on –Leu SDO plates. We followed transformation methods as per Yeast Protocols Handbook (Clontech). However, our efficiencies reached a maximum of 2.50 $x10^4$ CFU/μg DNA as per recommended methods. High efficiency transformations were obtained using these changes: initial overnight growth of Y187 yeast growing in YPDA at 30 °C shaking at 250 RPM was limited to 15 hours, resulting in a ¼ dilution measured via OD600 to be around 0.8. Under these conditions, 13 mL of concentrated yeast were added to 400 mL of YPDA so that the OD600 measurement was just below 0.2. This was given 3.5 hours to grow to an OD600 of no more than 0.5. Though the Yeast Two Hybrid protocol stipulates that an OD600 of 0.6 is tolerable, transformations performed at a final OD600 of 0.6 were found to have significantly reduced efficiencies. Finally, after transformation with plasmid DNA and immediately prior to plating, we found resuspension in 1X TE (as recommended) to result in lowered yields. Instead, we grew up transformed yeast resuspended in either 2X YPDA or YPDA plus (1.0 mL) for 1.5-2.0 hours at 30 °C and 200 RPM shaking. After this additional growth period, yeast were resuspended in 0.5 mL 0.9% NaCl, plated on –Leu SDO plates and allowed to grow for 5 days, resulting in a transformation efficiency of 1.90 $x10^5$ CFU/μg DNA.

Library scale transformation:

Prior to performing our library scale transformation, we validated that Y187 indeed does take up linearized vector plasmid and cDNA fragments in order to generate a stable circularized plasmid. Using our optimized small-scale transformation protocol (above), we transformed linear pGATT7-Rec (0.5 μg) and linear pGADT7-Rec (0.5 μg) + control T fragment cDNA (0.1 μg). Plated on –Leu SDO plates, the linearized plasmid yielded a transformation efficiency of 1.1 $x10^4$ CFU/μg, whereas the plasmid with the insert had a transformation efficiency of 9.2 $x10^4$ CFU/μg DNA (when considering the plasmid as limiting reagent). This was roughly a ten-fold increase, as expected. The colonies from the linearized plasmid are expected, as linear plasmids frequently recombine with host-yeast genomic DNA.

Next, we performed a series of co-transformations and test-matings (according to protocol in Matchmaker Gold Yeast Two-Hybrid System User Manual, Clontech) to verify that our bait, pGBKT7-SAX-7, could successfully be expressed in our AH109 reported bait strain. Oddly, pGBKT7-SAX-7 and pGADT7-UNC-44 or pGADT7-STN2 (known interactors) would not trigger reporter expression on quadruple drop out (QDO) –LTHA plates when co-transformed or when mated. This was contrary to what was previously reported (Zhou, 2008). We assumed that these may not be extremely strong interactors, and are not viable under the harshest stringency of the quadruple drop out conditions as the adenine reporter system in AH109 is only triggered by stable bait-prey interactions. We repeated a mating of pGBKT7-SAX-7 and with pGADT7-UNC-44 or pGADT7-STN2 and plated on triple drop out (TDO) –LTH plates. These interactions were demonstrated, as is summarized in Table 1. This shows that SAX-7 can be expressed in AH109, and that the SAX-7 and Gal4 BD (DNA binding domain) could successfully activate the HIS

reporter when expressed with a prey-hybrid interactor protein containing the Gal4 AD (activation domain).

Table 1: Growth from mating of Sax-7 in AH109 with respective prey in Y187.

	1/1,000 dilution, -LT plates	1/1,000 dilution, -LTH plates
STN-2	**124 colonies**	**108 colonies**
UNC-44	**598 colonies**	**470 colonies**

Given that SAX-7 was shown to be successfully expressed in our bait strain, we proceeded to transform our library into the AH109 bait strain using the protocol from the Yeastmaker™ Yeast Transformation System 2 User Manual (Clontech). We modified the protocol to use our previously optimized growth windows from the small-scale transformation protocol (previous page). Transformed DNA consisted of our 2 μg *C. elegans* library combined with 3 μg of pGADT7-Rec, as well as the recommended 200 μg of salmon sperm carrier DNA (boiled prior to transformation for 5 minutes in order to obtain single stranded DNA). Transformed yeast with library were streaked with sterile glass beads on 100 plates, 150 mm –Leu SDO +kanamycin (50 μg/L), and grown for 5 days at 30 °C. Transformation efficiency was determined by the concurrent plating of a 1:100 dilution on a 100 mm plate with –Leu selectable marker. As 150 colonies grew on the indicator plate, we conclude our transformation efficiency was 7.5 $x10^5$ CFU/μg DNA. Colonies on the 100 plates were washed with YPDA/25% glycerol + kanamycin, combined and stored at -80C for a total yield of 300 mL of prey yeast containing library cDNA. Using hemocytometer, we determined our final concentration was 1.585 $x10^9$ cells/mL, significantly above the required 7.0 $x10^7$ cells needed for one interaction screen.

Mating and Interaction Screen:

A yeast mating was performed as per the protocol of the Matchmaker Gold Yeast Two-Hybrid System User Manual. AH109 expressing the bait strain, pGBKT7-SAX-7, was grown up for 16 hours in –Trp medium, reaching an OD600 of 1.123. This bait was pelleted, combined with our Y187 library strain (1mL), and grown at 30°C at 30 RPM for 20 hours in 2x YPDA medium. While yeast zygotes were identified via light microscopy at 20 hours, an additional 4 hours of mating was provided in order to produce an optimum yield of zygotes containing both plasmids (Figure 3). Mated yeasts were then plated on 50 plates, 150 mm containing –LTH selective media, and grown for 5 days at 30°C. Concurrently, we plated mated yeasts on –L, -T, and –LT media in order to determine mating efficiencies, as shown in Table 2. As the limiting factor to this mating screen is the prey library strain, 10 μL of our library was extracted prior to combining the strains and plated at a 1/10,000 dilution on a –Leu plate: roughly 1,200 colonies grew, providing a library titer estimate of 1.2 $x10^8$ cells/mL.

Figure 3: Microscopy identification of mating yeast.

This image was taken after 20 hours of bait strain AH109 and prey strain Y187 interaction. Yeasts were imaged at 630x using a light microscope. At this time, both "Mickey Mouse" and cloverleaf features of typical yeast zygotes were identified. The three-leaf structure, as indicated by the arrow, suggests the population was undergoing successful mating.

Table 2: Plating results for determining mating efficiency

	# of Colonies (Dilution: 1/10,000)	Viability
-Leu plates	202	Viability of Prey: $2.5 x10^7$
-Trp plates	2300	Viability of Bait:

		2.9×10^8
-Leu/Trp plates	25	Viability of Diploid: 3.2×10^6

As the limiting factor to the mating screen was our prey library, we estimated the mating efficiency by calculating the Diploid Viability/ Prey Viability = 12.8 % mating efficiency. On the 50 –LTH plates, we found an average of 80 colonies per plate, suggesting we found roughly 4,000 colonies expressing potential interactors. Many of these are likely identical colonies and will need to be reduced down to 1 copy during stringency testing.

Discussion and Future Directions:

This project generated far more interacting colonies than was initially anticipated. The reasons for these results can be attributed to having a wide range of cDNA fragments cloned using random hexamer primers, high transformation efficiency, and adequate mating efficiency. Specifically, we would have liked to have gotten further and characterized several of these protein interactors. For future directions, we shall analyze our results using higher stringency screening. This includes replica plating onto –LTH plates containing α–gal, 3-AT to reduce leaky histidine expression (which may result in false positive interactions), and subsequent plating onto –LTHA as the highest stringency assay. From there, restriction digests of the cDNA insert shall be performed to eliminate colonies with identical inserts (Koolonin, 2008). We shall then use DNA sequencing to begin analyzing fragments: as yeast use leaky scanning, the frame of our random primer generated cDNA need not be considered as plasmids with random primer inserts in yeast can detect valid interactions that are not necessarily found with full length open reading frames (Hastie, 2007). Thus, it follows logically to begin with analyzing sequences that have domains that can be recognized by the FERM, PDZ, or ankyrin binding domains (Chen, 2010). Of course, there are other potential and uncharacterized binding domains between these three domains, so proteins that can be recognized by even one of these domains may have recognition sites with novel interactions and functions.

Improvements to this protocol would be primarily through considerations regarding how the cDNA was generated. In particular, several reverse transcription reactions catalyzed by the MMLV reverse transcriptase failed, which we ascribe to low concentration of poly(A) enriched mRNA. Concentrating the enriched mRNA prior to the RT-PCR reaction may further improve the quality of the library. In regard to the mating protocols, it seems odd that previously characterized interactions could not be replicated on –LTHA plates. This suggests the AH109 adenine reporter system requires very strong interactions. This provides us with a method for specifically testing binding partners for strong interactions. Additionally, it may be valuable to reconsider the nature of the interaction previously found, namely, of STN-2 and UNC-44 with SAX-7.

Additional features not directly mentioned in this report are that we successfully generated the equivalent of 300 copies of a library. Not only can 300 separate screens be performed (for SAX-7 or otherwise), but can be done without first having to amplify the library in *E. coli*. This prevents losing interactors that have mRNA expressed at low levels in *C.elegans*, since any amplification will skew the distribution of a library for transcripts that are more predominant. Considering that we had a diploid viability of 3.2×10^6, the number of interactions that can be screened and the scale at which matings can be performed to allow for interaction screens to be several orders of magnitude more comprehensive than typical co-transformation experiments.

Special Thanks:

Thank you to the Chen lab for providing the equipment, space, and intellectual brilliance to help a novice scientist such as myself achieve those moments of outstanding happiness in the biological sciences—times that can only be appreciated after repeated rounds of frustration, heartbreak, and the ever persistent need for more-supplies. In particular, thank you to Dr. Chen for her insight in overseeing this project, and her outstanding and ever-present patience.

Thank you to the UROP committee for supporting my research, and for encouraging excellent student research throughout the College of Biological Science. Your support has helped many students, including myself, have the opportunity to develop both skills and an appreciation for research that extends far beyond the limits the class. I hope that what I have learned will help in the years ahead as I move on to become a full-time researcher.

Reflective statement on UROP experience:

This project was far more comprehensive than expected. Specifically, we had several setbacks: our library was reduced to 1 μg when we ran it through the CHROMA SPIN columns, resulting in the need to generate more library. Next, the protocol for transforming yeast was atrocious. As we only had one shot with reagents, and as buying supplies to construct another library would have cost over $1,000, Dr. Chen and I made an executive decision to **do things right**. Thus, we spent over a month and a half optimizing protocols that ideally would have been provided with the kit. Well, if anybody should care to build their own library (as opposed to "borrowing" some of ours), these modified protocols detail exactly how to construct a library in Y187 yeast. Indeed, we learned far more about Y187 yeast and the AH109 expression system than anticipated. Because the stringency on –LTHA plates was demonstrated to be very high, and because alpha-gal is known to reduce yeast growth, we made an egregiously extensive screen of interactors—over 4,000! Clearly these numbers will need to be reduced through several rounds of selectivity screening.

We completed the first 3 objectives of this experiment outlined in the initial proposal, but never reached the characterization phase of identified interactors. In retrospect, characterizing interactors thoroughly could take years. We shall continue with the research in upcoming months, focusing almost exclusively on interactors that have FERM, PDZ-recognition, or ankyrin domains. This paper shall be uploaded to the U of M Digital Conservancy so peers and researchers have access to these data.

Though this project had moments that were very frustrating, with unanticipated problems that took over a month to resolve, this experience has shown me what true research actually entails. In retrospect, I take great pride having successfully completed the construction of a yeast two hybrid library, something that is mentioned in textbooks but should not be considered a trivial task. This research is an excellent foundation for learning about molecular interaction

studies, something that will help me pursue (graduate) research in neuroscience involving signal transduction pathways and studying protein interaction networks.

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